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Tiazofurin-Induced Autosecretion of IL-6 and Hemoglobin Production in K562 Human Leukemia Cells

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Previous reports have established the synthesis of interleukin-6 (IL-6) and IL-6 receptors (IL-6R) in several human leukemia cells and found that IL-6 and the IL-6R could be expressed in cell lines with erythroid/megakaryocytic features. IL-6 is a pleiotropic cytokine involved in megakaryocytic differentiation. The finding that endogenous IL-6 levels in serum increased after 5-fluorouracil (5-FU) treatment suggests that IL-6 may play some role in the recovery of hematopoietic systems. This observation may assist the understanding of erythroid regeneration caused by antineoplastic agents such as tiazofurin. Tiazofurin inhibits the activity of IMP dehydrogenase. Its exposure to K562 cells at 10 μM tiazofurin stimulates erythroid differentiation. Stimulation of cells with tiazofurin gave a significant increase in IL-6 production. Its levels were quadrupled after 2 days of culture. Tiazofurin also caused a trivial reduction in the percentage of cells with the IL-6R. This evidence implies that tiazofurin produced no significant effect on the IL-6R. Tiazofurin also increased the percentage of benzidine-positive cells representing hemoglobin production, confirmed by GpA expression. We concluded that IL-6 is rate limiting in regard to hemoglobin production and that IL-3 could be used for clinical benefit to stimulate erythropoiesis and synergize with tiazofurin. Am. J. Hematol. 54:301–305, 1997.

Key words: interleukin-6; interleukin-6 receptor; tiazofurin; megakaryocytic; erythroid differentiation

INTRODUCTION

Interleukin-3 and -6 (IL-3 and IL-6) levels act synergistically to support the proliferation of hematopoietic progenitors [1]. IL-3 can exert its stimulatory action on erythropoiesis but cannot trigger hematopoietic stem cells (HSCs) to progress from G_0 into active cycling [2,3]. IL-6, however, puts HSCs into the cell cycle by stimulating quiescent progenitors to enter the S phase or into active cycling [4,5]. It provides a unique nexus to IL-3. IL-6 enhances the sensitivity of multipotential progenitors to IL-3. It does this by increasing the number of interleukin-3 receptors (IL-3Rs) or by upregulating the affinity of receptors to IL-3 on hematopoietic progenitors [6]. Indeed, IL-6 induces both erythroid and megakaryocytic development when combined with IL-3 and plays an important role in the production of HSCs at the level of the primitive stem cell [7,8]. We examined the erythroid and megakaryocytic differentiationcaused by IL-6. In our approach, we used the human leukemia cell line K562 because of its capacity to undergo erythroid and megakaryocytic differentiation by inducing agents [9].

Abbreviations: IL-6, interleukin-6; IL-6R, interleukin-6 receptor; IL-3, interleukin-3; IL-3R, interleukin-3 receptor; HSC, hematopoietic stem cell; GpA, glycophorin A; Hb, hemoglobin; TZF, tiazofurin; IMP, inosine monophosphate; Epo, erythropoietin; ELISA, enzyme-linked immunoabsorbent assay.

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K562 cells are able to produce and secrete IL-6 in untreated cultures, making these cells an excellent model to study this parameter. On the other hand, it is noteworthy that transcripts of IL-3 mRNA were not detected in K562 cells, along with no autocrine secretion of IL-3 [10]. Therefore, it was not necessary to evaluate increasing levels of endogenous IL-3. In culture, fetal bovine serum (FBS) contains ~1 U/ml of IL-3, which gives IL-6 an opportunity to work synergistically with IL-3 [11]. Along with IL-6, IL-6R was another parameter that we wanted to evaluate. It is virtually undetected in uninduced cells but is expressed by cells stimulated by phorbol esters [12]. Phorbol esters have been shown to act by activating protein kinase C, thereby enhancing the accumulation of IL-6 receptor mRNA in K562 cells [13]. In particular, IL-6R has been demonstrated to be predominant on megakaryocytes and will be used to determine megakaryocytic differentiation [14].

In K562 cells, tiazofurin (2-β-D-ribofuranosyl-4-thiazolecarboxamide) at 10 μM stimulated erythroid differentiation as shown in Phase II clinical trials for the treatment of acute myelogenous leukemia [15,16]. The mechanism of its erythroid stimulation is unknown. Tiazofurin is an antineoplastic agent that inhibits IMP dehydrogenase activity and thus retards leukemia cell growth [17]. Here, we have stimulated cells with 10 μM tiazofurin and have observed elevated IL-6 levels. IL-6 production is concomitant with hemoglobin production during erythroid differentiation.

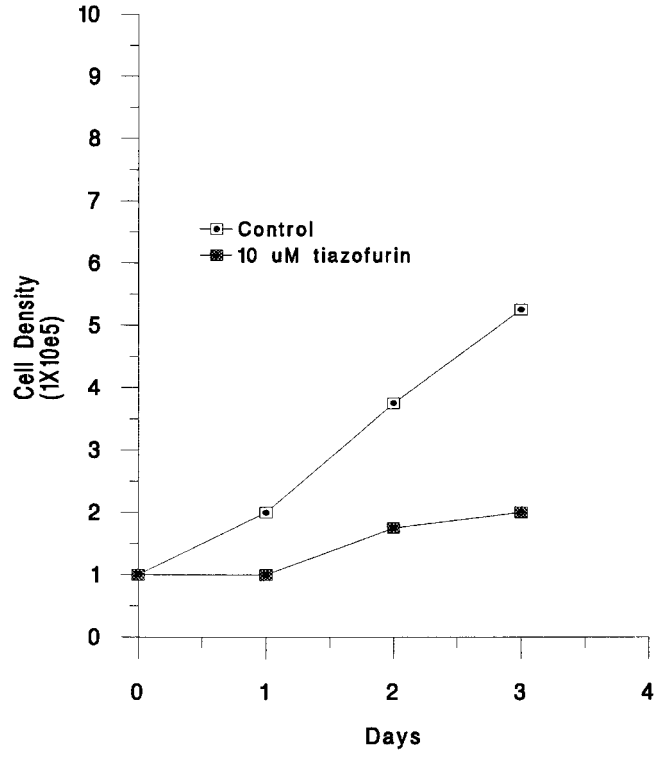

Fig. 1. Growth kinetics of K562 cells in response to 10 μM tiazofurin treatment. The means of quadruplicates are given (SEM < 10%, *P* < 0.05). Cell viability was always >90%, as monitored by trypan blue exclusion.

MATERIALS AND METHODS

Cell Culture

K562 cells (ATCC, Rockville, MD) were maintained in RPMI 1640 medium with 25 mM Hepes buffer and L-glutamine (Gibco-BRL, Grand Island, NY) with 0.5% gentamicin (Gibco-BRL) and 10% FBS (Gibco-BRL) at 37°C. Cells were suspended in culture at a concentration of 1×10^5 cells/ml. They were then induced at day 0 with 10 μM tiazofurin (National Cancer Institute, Bethesda, MD). Cell densities (cells/ml) were determined with Coulter Counter Analysis (Coulter Electronics, Hialeah, FL) and checked with a hemocytometer for trypan blue exclusion assay, performed daily. Cell cultures were subcultured during exponential growth at 1×10^5 cells/ml by dilution.

IL-6 ELISA

Seven standards, within the range of 300–3.13 pg/ml, were used as controls for IL-6 quantitation by enzyme-linked immunosorbent assay (ELISA). Cell cultures were treated with 10 μM tiazofurin for 2 days. Cells were frozen at −20°C overnight, then thawed to lyse cells. Culture supernatant preparations were evaluated in triplicate using microtiter plate strips (R&D Systems, Minneapolis, MN). These samples were combined with RD5A Assay diluent and incubated at room temperature for 2 hr. The microtiter plate was washed four times before adding the IL-6 conjugate, covered with an adhesive strip, and incubated again. The wash/aspiration process was repeated. Colorimetric agents were added to allow for absorbance. The optical density was determined using a spectrophotometer set at 450 nm with a wavelength correction at 540 nm. The IL-6 concentration for each sample was evaluated from a standard curve, using IL-6 controls (300–3.13 pg/ml). The secreted IL-6 pg/1×10^6 cells quantity for presentation purposes was obtained by dividing the picograms per milliliter (pg/ml) value by cell density. Experiments were performed by the R&D Systems procedure provided.

Hemoglobin Detection

The percentage of benzidine-positive stained cells was determined by counting at least 500 cells by the wet benzidine method. Positive cells were stained blue, and negative cells were stained yellow under a hemotocytometer. The benzidine stain used was prepared by adding 5 μl of 30% hydrogen peroxide to 1 ml of stock solution of 0.2% benzidine (Sigma Chemical Co., St. Louis, MO)/0.5% acetic acid; 50 μl of this solution was used for each 50 μl of cell suspension. This procedure was done as previously described [18].

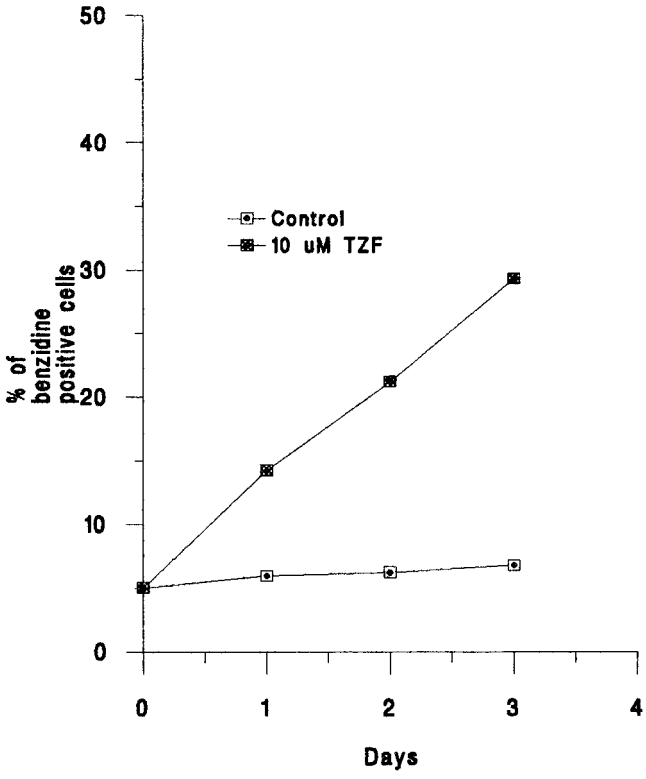

Fig. 2. The percentage of benzidine positive cells induced by 10 μM tiazofurin. Means of triplicates are given (SEM < 5%, $P < 0.01$). Benzidine-positive cells are an indication of the presence of hemoglobin.

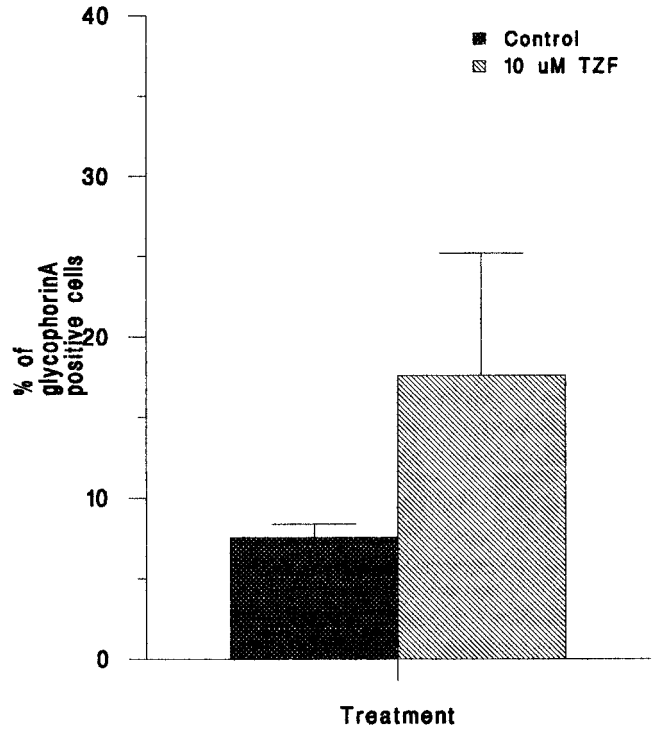

Fig. 3. Percentage of cells with specific fluorescence of the GpA in response to treatment. Means of triplicates are given. SEM values are shown in graph and $P < 0.05$.

IL-6R Expression

Experiments were done using indirect immunofluorescence with MT18 antibody (a generous gift of Tadamitsu Kishimoto, Osaka University, Osaka, Japan) and a secondary antibody IgG-PE (Sigma). The wash buffer used contained PBS with 0.01% sodium azide/1% bovine serum assay (BSA) (EiA grade). Aliquots of 5×10^5 cells were obtained from the control and tiazofurin treatment samples. Cells were stained with the MT-18 antibody. The cells designated as the control were used to determine nonspecific fluorescence. Cells were incubated with 30 μl of MT-18 for 25 min, combined with 1 ml of wash buffer, centrifuged for 5 min at 1500 rpm, then aspirated and vortexed. Both sets of cells were incubated with 20 μl of whole IgG-PE labeled for 25 min, then combined with 1 ml of wash buffer. Samples were then stored at 4°C until analyzed with the FACScan flow cytometer using LYSYS II software v.1.1 to determine specific fluorescence on gated K562 cells.

Glycophorin A Cytofluorometric Assays

The monoclonal antibodies (mAb) used were GpA-PE (Gentrak, Amersham, NY) and IgG-PE (Sigma) to determine the presence of GpA^+ cells. For each determination, 1×10^6 cells were obtained from culture. Fluorescence analysis was performed by FACScan flow cytometer (Becton-Dickinson Immunocytochemistry Systems, San Jose, CA) with a laser excitation set at 488 nm, using the LYSYS II software program v.1.1.

Statistical Analysis

Statistics were determined using the Instat program, as displayed by standard error bars (Graph Pad Software, San Diego, CA).

RESULTS

Effects of Tiazofurin on Cell Growth and Erythroid Differentiation

The growth kinetics of K562 cells are shown in Figure 1. There was an observed doubling time of 24 hr. There was no change in the doubling time of 24 hr during the first 2 days of culture; 10 μM tiazofurin treatment appeared to cause an arrest of growth at 24 hr. The doubling time in response to tiazofurin treatment was augmented to 48 hr.

There was an increased percentage of benzidine-positive stained cells, an indication of the presence of hemoglobin [18]. The increase in erythroid differentiation was highly significant, as shown in Figure 2. In particular, glycophorin A has been used in K562 cells to detect erythroid differentiation [19]. Our observations

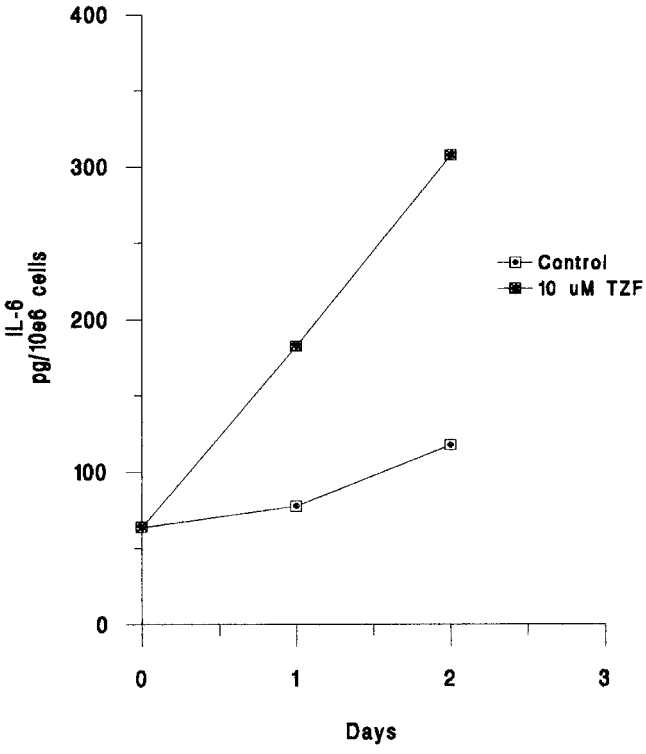

Fig. 4. Secretion of IL-6 in response to 10 μM tiazofurin using ELISA. Means of quadruplicates were given (SEM < 10%, $P < 0.05$). The IL-6 pg/1 × 10^6 cells quantity was determined by the pg/ml and the cell density values.

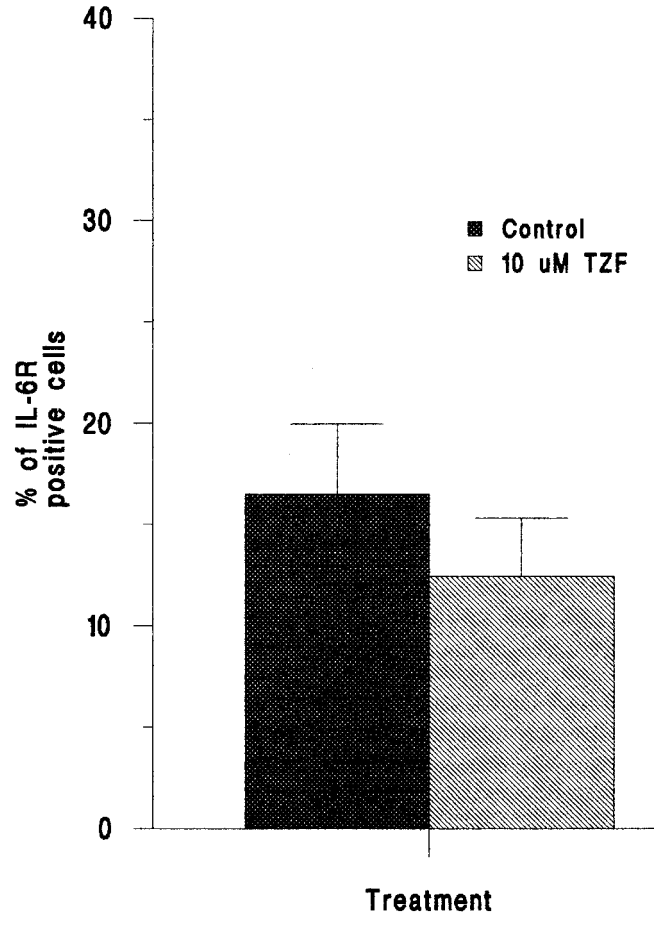

Fig. 5. Percentage of cells with specific fluorescence of the IL-6 receptor in response to treatment. Means of triplicates are given. SEM values are shown in graph and $P > 0.05$.

displayed an increase of GpA expression at 24 hr up to 17% signifying phenotypic expression of erythroid differentiation (Fig. 3). The degree of expression correlated well to the percentage of Hb producing cells of 15% at 24 hr. Thus, erythroid differentiation was consistently elevated by tiazofurin.

IL-6 Autosecretion and IL-6R Expression

Autosecretion of IL-6 was shown to be elevated with tiazofurin (Fig. 4). This agent caused a threefold increase of secreted IL-6 after each interval. Stimulation of cells with tiazofurin gave a significant increase in production of the cytokine. Its levels were quadrupled after 2 days of culture. By increasing HSCs, IL-6 seems to be rate limiting in regard to hemoglobin-producing cells.

Indeed, IL-6R has been shown to exhibit inducibility in K562 cells, and the presence of the IL-6R is found in megakaryocytes only [20]. Because IL-6 is a pleiotropic cytokine involved in megakaryocytic and erythroid differentiation, we wanted to determine its lineage specificity by IL-6R detection [5,6]. In our evaluation, a trivial decrease in IL-6R expression was noted (Fig. 5) after one day of culture with Tiazufurin: Expression of IL-6R in untreated K-562 cells decreased significantly (fourfold from day 1) by day 2, whereas expression on Tiazofurin-treated cells appeared to drop only slightly. Tiazofurin was unable to induce megakaryocytic differentiation, strongly suggesting that the stimulation caused by IL-6 is erythroid lineage specific.

DISCUSSION

The finding that endogenous IL-6 levels in serum was increased after 5-fluorouracil (5-FU) treatment suggests that IL-6 may play some role in the recovery of hematopoietic systems [21]. Our observations were highly consistent with hematopoietic regeneration. We were able to detect elevated IL-6 levels with concomitant growth inhibition during tiazofurin treatment. Our evidence is consistent with the accumulation of IL-6 being relative to hemoglobin synthesis, and possibly a rate-limiting factor for erythroid progenitors.

By augmenting HSCs, IL-6 can increase the amount of erythroid progenitors by increasing the stem cell pool. We contend that IL-3 can combine with IL-6 to stimulate benzidine-positive erythroid cells. IL-6 can also synergize with Epo to increase the number of erythroid pro-

genitors more so than Epo alone [22]. Furthermore, IL-6 represents the impetus for HSCs to become active allowing further erythroid proliferation and maturation. We have shown that IL-6 induces differentiation down the erythroid and not the megakaryocytic lineage. Future studies directed at evaluating the effects of IL-3 and Epo receptors by tiazofurin would help elucidate the mechanism of erythroid development in K562 cells. In conclusion, the stimulation of erythropoiesis by tiazofurin could have possible synergism between rhIL-3 in clinical situations.

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REFERENCES

1. Ikebuchi K, Wong GG, Clark SC, Ihle JN, Hirai Y, Ogawa M: Interleukin 6 enhancement of the interleukin 3-dependent proliferation of multipotential hematopoietic progenitors. Proc Natl Acad Sci USA 84:9035–9042, 1987.
2. Gabbianelli M, Pelosi E, Labbaye C, Valtieri M: Reactivation of HbF synthesis in normal adult erythroid bursts by IL-3. Br J Haematol 74:114–117, 1990.
3. Suda T, Suda J, Ogawa M, Ihle JN: Permissive role of interleukin 3 (IL-3) in proliferation and differentiation of multipotential hematopoietic progenitors in culture. J Cell Physiol 124:182–187, 1985.
4. Leary AG, Ikebuchi K, Hirai Y, Wong GG, Yang YC, Clark SC, Ogawa M: Synergism between interleukin-6 and interleukin-3 in supporting proliferation of human hematopoietic stem cells: Comparison with interleukin-1α. Blood 71:1759–1765, 1988.
5. Ikebuchi K, Ihle JN, Hirai Y, Wong GG, Clark SC, Ogawa M: Synergistic factors for stem cell proliferation: Further studies of the target stem cells and the mechanism of stimulation by interleukin-1, interleukin-6 and granulocyte colony-stimulating factor. Blood 72:2007–2014, 1988.
6. Kishimoto T: The biology of interleukin-6. Blood 74:1–10, 1989.
7. Rennick D, Jackson J, Yang G, Wideman J, Lee F, Hudak S: Interleukin-6 interaction with interleukin-4 and other hematopoietic growth factors to selectively enhance the growth of megakaryocytic, erythroid, myeloid and multipotential progenitor cells. Blood 73:1828–1835, 1989.
8. Hirata Y, Taga T, Hibi M, Nakano M, Nakano H, Tadamitsu K: Characteristic of IL-6 receptor expression by monoclonal and polyclonal antibodies. J Immunol 143:2900–2906, 1989.
9. Sutherland JA, Turner AR, Mannoni P, McGann LE, Ture JM: Differentiation of K562 leukemia cells along erythroid, macrophage and megakaryocyte Lineages. J Biol Respir Mod 5:250–262, 1985.
10. Barber KE, Crosier PS, Purdie KJ, Buchanan JM: Human interleukin 3: Effects on normal and leukemic cells. Growth Factors 2:101–104, 1989.
11. Koike K, Nakahata T, Takagi M, Kobayashi T, Ishiguro A, Tsuji K, Naganuma K, Okano A, Akiyama Y, Akabene T: Synergism of BSF-2/interleukin 6 and interleukin 3 on development of multipotential hematopoietic progenitors in serum-free culture. J Exp Med 168:879–890, 1988.
12. Navarro S, Mitjavila MT, Katz A, Doly J, Vainchenker W: Expression of interleukin-6 and its specific receptor by untreated and PMA-stimulated human erythroid and megakaryocytic cell lines. Exp Hematol 19:11–17, 1991.
13. Ray A, Tatter SB, Santhanam U, Helgott DC, May LT, Sehal PB: Regulation of expression of interleukin-6. Ann NY Acad Sci 557:353–362, 1989.
14. Jokai J, Ryo R, Yasunga M, Sugano W: Detection of mRNA in megakaryocytes using in situ hybridization. Rinsho Byori 40:841–847, 1992.
15. Olah E, Natsumeda Y, Ikegami T, Zsofia H, Paulik E, Kremmer T, Hollan SR, Sugar J, Weber G: Induction of erythroid differentiation and modulation of gene expression by tiazofurin in K562 leukemia cells. Proc Natl Acad Sci USA 85:6533–6570, 1985.
16. Tricot GJ, Hiremagalur NJ, Nichols CR, Pennington K, Lapis E, Weber G, Hoffman R: Hematological and biochemical action of tiazofurin in a case of refractory acute myeloid leukemia. Cancer Res 47:4988–4991, 1987.
17. Lui MS, Faderan MA, Liepnieks JJ, Natsumeda Y, Olah E, Hiremagalur NJ, Weber G: Modulation of IMP dehydrogenase activity and guanylate metabolism by tiazofurin. J Biol Chem 259:5078–5082, 1984.
18. Orkin S, Swan D, Leder P: Differentiation in erythroleukemia cells and their somatic hybrids. Proc Natl Acad Sci USA 72:98–102, 1975.
19. Rowley PT, Farley BA, LaBella S, Giuliano R, Leary JF: Single K562 human leukemia cells express and are inducible for both erythroid and megakaryocytic antigens. Int J Cell Cloning 10:232–240, 1992.
20. Navarro S, Debili N, Le Couedic JP, Klein B, Breton-Gorius J, Doly J, Vainchenker W: Interleukin-6 and its receptor are expressed by human megakaryocytes: In vitro effects on proliferation and endoreplication. Blood 77:461–471, 1991.
21. Takasuki F, Okano A, Suzuki C, Miyasaka Y, Hirano T, Kishimoto T, Ejima D, Akiyama Y: Interleukin 6 perfusion stimulates reconstitution of the immune and hematopoietic systems after 5-fluorouracil treatment. Cancer Res 50:2885–2890, 1990.
22. Wei DM,Wang LX, Chen PS: Effect of recombinant human growth factors: Recombinant human interleukin-6, recombinant human granulocyte-macrophage colony-stimulating-factor and recombinant human erythropoietin on normal human hematopoietic progenitors cells. Chin J Intern Med 33:298–301, 1994.